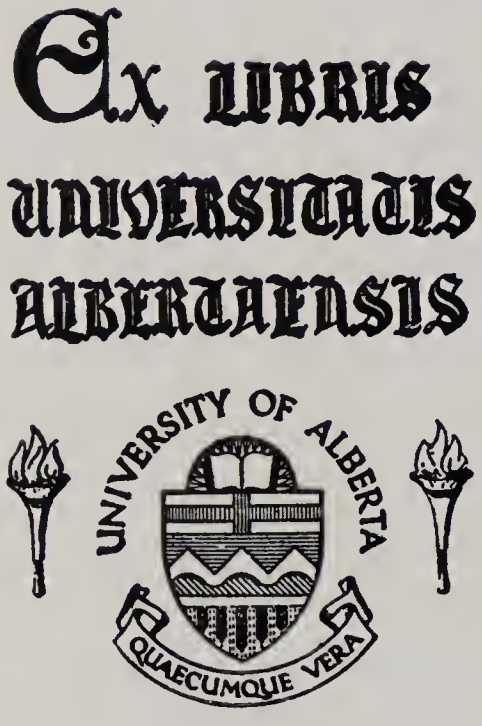

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SEX HORMONES AND PROLACTIN IN PHALAROPES IN RELATION TO PLUMAGE AND BEHAVIORAL SEX DIMORPHISM

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN

PARTIAL FULFILMENT OF THE REQUIREMENTS OF

THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHYSIOLOGY

by

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Sex Hormones and Prolactin in Phalaropes in Relation to Plumage and Behavioral Sex Dimorphism", submitted by SU CHIAU CHENG in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

A study was undertaken to investigate the endocrine factors involved in the natural occurrence of more colorful nuptial plumage in female than male, as well as the sexual reversal in courtship and aggressive behavior and incubation, in phalaropes. Five steroids were separated from the gonadal extracts of Wilson's phalaropes and five other species of birds by the method of paper chromatography followed by colorimetric determination. Pituitary prolactin content of male and female breeding phalaropes was determined by the pigeon crop-sac test and by cytological study. Relative pituitary weights of breeding phalaropes and two other species of birds were also recorded.

The levels of gonadal steroid secretion was seen to be reflected by the difference in gonadal steroid hormone content of breeding and non breeding mallards. Relatively higher ovarian testosterone content per unit body weight than in the testes of conspecific male phalaropes, and a higher ovarian testosterone/estradiol ratio in phalaropes than in the other species examined were observed. These observations taken in conjunction with the experimental finding of others that androgens but not estrogens can induce the formation of nuptial feathers in phalaropes of either sex explain the formation of the brighter nuptial

plumage in female than male phalaropes. On the basis of the general dependence of aggressive behavior on androgens in most birds, the observations on gonadal steroid hormone content also explain the greater aggressive behavior of female than male phalaropes.

Observation of a higher estrogen content in testes of breeding than non breeding mallards supports the hypothesis of estrogen control of the male eclipse plumage in mallards.

Prolactin like action shown only by the male not female pituitaries of Wilson's phalaropes together with the observation of a greater number of acidophils per unit area of adenohypophysis in male than in female Northern phalaropes explain the fact that only the male phalaropes form incubation patches, incubate and brood the young, since it has been demonstrated that only the synergistic action of prolactin and testosterone but not estrogen can induce the formation of incubation patches in phalaropes, and that prolactin induces the broodiness in certain birds. Indirect evidence for this view came from the observation of sex differences in pituitary weights of Wilson's phalaropes, Redwinged Blackbirds and Killdeers in relation to the incidence in these species of brood patch formation in one or both sexes.

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GLOSSARY OF CERTAIN ORNITHOLOGICAL TERMS USED IN THIS THESIS

Circling: A display movement of male domestic fowl in which the cockerel struts in an imposing posture around the hen.

Incubation patch: An area on the ventral body surface of most birds from which the feathers are shed just before the eggs are laid and in which the skin becomes hypervascularised and odematous. This patch denuded of feathers is brought into contact with the eggs during incubation.

Territory, territorial behavior: The territory is a piece of ground defended by a male bird (in typical cases) against intrusion by other males of its own species, by means of song, special calls, threats or actual attacks. Within this area (after a male has secured a female) mating and later nesting take place. Territorial behavior is essentially the defence of the territory.

Fig. A. A pair of Wilson's Phalaropes, the more colorful female is at the right, the dull colored male at the left.

I. Introduction:

The phalaropes, a family of three species of shore birds, the Red phalarope (Phalarope fulicarius), Northern phalarope (Lobipes lobatus), and Wilson's phalarope (Steganopus tricolor) show a peculiar reversal of the frequent sex dimorphism often found in birds in that the females are larger and have a more brightly colored breeding plumage than the males. These features are shared only with the painted snipe and the button squails. Female phalaropes have been observed to be pugnacious against rivals of the same sex by threats or attacks on other females thus keeping the latter away from the males they have selected. They obviously take the leading part in courtship (1, 2). Males only show occasional aggressive behavior somewhat later in the breeding season towards either males or females, although copulation can be initiated by either sex (2). The only species, the Northern phalarope, known to show territorial behavior shows this only in females (3, 4).

Ample evidence has been accumulated showing that in birds aggressive as well as male sexual behavior are generally induced by androgens (5, 6), while estrogens have a tendency to lower aggressiveness (7). These facts led us to suspect that female phalaropes might produce unusually large amounts of androgens compared to other female birds in the breeding season and that male phalaropes might

be good estrogen or poor androgen producers. The present study was thus carried out to determine the gonadal content in both sexes of Wilson's phalaropes in comparison with some other birds in which the male is the dominant sex. Gonadal extracts were subjected to paper chromatography and colorimetric determination of the steroids thus separated.

Another unusual feature of phalarope reproduction is that nest building is only performed by the male phalarope which developes an incubation patch, incubates and broods the newly hatched young. The females leave the nesting areas long before the eggs hatch though the disturbed females show distraction display from the nest during the egg laying period. (2). It has been demonstrated that in certain fringillids, passerine birds in which only the females develop incubation patches and incubate, incubation patch formation depends on estrogen and prolactin action (8). Also, prolactin can induce broodiness both in laying and non-laying hens (9). In phalaropes, Johns and Pfeiffer (10) have shown that testosterone in combination with prolactin produce incubation patches in both male and female Wilson's phalaropes and Northern phalaropes and that estradiol alone or given with prolactin is ineffective. The failure of formation of incubation patches in normal female phalaropes was thus thought to be due to a deficiency of prolactin if the hypothesis that female phalaropes

are good androgen secretors were true. Therefore, assays of pituitary prolactin content in both sexes of phalaropes accompanied with a histological study of the pituiaties were performed.

II. Literature survey:

A. Hormones and Aggressive and Sex behavior in Birds.

It has long been recognized that there is a close relationship between gonadal activity and the sex behavior in birds (11). Besides, it is a common knowledge that the males of many species are more aggressive than the females and that aggressiveness is increased during the breeding season. Thus, a stimulating action of gonadal hormones on aggressive as well as sex behavior was postulated and studies have been carried out extensively demonstrating the real existence of this relationship. Also, a particular gonadotrophin is involved in one case in the induction of aggressive behavior.

1. Hormones and aggressive behavior.

(a) Gonadal hormones and aggressive behavior.

Aggressive behavior in birds is manifested in the defense of a territory around the breeding place to subserve mating, nesting and feeding uses, and in the attainment of dominance within a social group. It is also a step toward the achievement of reproduction by defending the mate against rivals.

In 1938, Hamilton (12) injected young male chicks with 0.5 mg of testosterone propionate daily for 27 days starting from the second day after hatching. These chicks showed aggressiveness, cock-like fighting and wing-flapping. Since then this relationship has been abundantly demonstrated. Noble & Zitrin (13) obtained the identical behavior as described by Hamilton after administration of testosterone propionate into young chicks. Further work of Hamilton (14) demonstrated that hens injected with androgen came to dominate control hens whom they had previously avoided, and there was an increased tendency of the injected hens to fight and peck at other hens. Allee et al (15) showed an advance in social status in low-ranking hens after injection of testosterone propionate. Similarly, in female canaries, following injection of testosterone the birds came to be in 1st, 2nd and 3rd positions instead of 4th, 5th and 6th (16). Subordinate individuals of ring doves became dominant following treatment with testosterone propionate (17). Moreover, testosterone propionate induced territory defense in month-old chicks, in immature birds and in adult females of the black-crowned night herons (18).

Valley quail males treated with testosterone showed a marked increase in pugnacity whereas no direct behavior response was detected in any of the birds treated with stilbestrol (19). In male Herring gulls, fighting occurs

frequently during the reproductive phase. Boss (20, 21) was able to show that continuous treatment with testosterone propionate from the ninth day of incubation, induced aggressiveness and interest in territorial defense in immature males and young male castrates. After discontinuation of male hormone injections immature males reverted to the juvenal type of behavior and estrogens did not modify the first year juvenal characteristic of behavior. Allee and Foreman (22) working on different breeds of Gallus gallus observed that the majority of the low-ranking hens receiving injection of testosterone propionate were able to overcome the social inertia within the flock and rose in their respective peck orders. Results from staged pair-contests demonstrated that individual aggressiveness had increased among all the androgen treated individuals. There were individual breed differences in the degree of response though no data on variation in threshold of effective stimulation had been reported.

In addition, some of the established results of gonadectomy in male fowls also lend support to the view that hormones from the testes have definite effects upon aggressive behavior. As is well known, early castration of male domestic animals often increases tractability, and Goodale (23) has described the reduced combativeness displayed by capons. Apparently, the above listed extensive investigation of the correlation of aggressive

behavior and androgen has well confirmed the view that androgens contribute to aggressive behavior and to the attainment of a dominant social position. There is no evidence to indicate that ovarian hormones exert similar effects. In fact, Allee and Collias (7) have discovered that administration of estrogen has a tendency to lower aggressiveness and social status in hens.

(b) Gonadotrophin and aggressive behavior.

In 1957, Davis (24) reported that castrated starlings maintained aggressive behavior and injections of testosterone failed to affect their social rank. It was also noted that aggressive behavior increased again in the fall even though gonadal weights remained at their minimal summer level. These facts suggested that in the starling androgens do not play a major role in determining aggressiveness but that other hormones might be involved. Mathewson (25) then showed that injection of Armor mammalian luteinizing hormone (LH) into subordinate male starlings influenced their aggressive behavior. Reversals of dominance occured within the first 10 to 15 minutes after injection. This rapid reaction suggests that circulating LH acts directly on the behavior centers in the brain rather than through gonadal hormones, in this species.

Davis (26) extended the work of effects of LH on aggressiveness under the conditions before and after castration. The data indicated that castrated birds nearly

always dominated their intact partners. When LH injections were given, there was clear-cut reversal of dominance. Since it is well established that the pituitaries of castrated animals contain more gonadotrophin than do those of intact animals, these experiments support the belief that in the starling LH rather than androgens controls aggressive behavior. So far, this is the only species in which aggressiveness has been shown to be influenced by LH. Ample evidence exists for androgen control as outlined above.

2. Hormones and sex behavior.

Courtship and mating activities of birds fall in the general category of sex behavior. Nest building, incubational behavior and care of the young after hatching are final steps in the reproductive process. Gonadal hormones have been clearly shown to exert an obvious control over the sex behavior of males and females (5).

The earliest evidence indicating the hormonal control of sex behavior is represented by a large number of observations revealing the close correlation between the condition of the gonads and the appearance of sexual activities. As the breeding season approaches gonad growth is accelerated and mating behavior becomes more intense (27). The relation of the size of the testes to reproductive activities of feral birds is described by Domm (28) as having been recorded as early as 1789 although the internal secretory function of gonads was not fully realized until

after the experiments of Shattock & Seligmann (29). It was then assumed that the sexual behavior changes noted as the gonads grow are brought about by the action of the internal secretion of gonads. Since then many lines of evidence have been reported supporting the theory of gonadal hormone control of sex behavior.

(a) Effects of removal of the source of the gonadal hormones by gonadectomy.

Goodale (31) reported that castrated cocks rarely crowed and they payed no attention to the hens. Ovariectomized hens did not visit the nests and they rarely cackled. They did not show any of the normal sexual reactions if approached by a cock. Similarly, domestic drakes and ducks, after complete gonadectomy showed no sexual behavior at all. Van Oordt and Jung (5) showed the loss of courtship behavior in the male ruff, following removal of the testes. In male bronze turkeys, castration resulted in elimination of sexual vacolization and in suppression of courtship and mating behavior (31). Carpenter (32) revealed that castration of the adult cock pigeon had depressing effects upon mating activity and precopulatory courtship became dissociated from the treading response to which it was normally closely related.

Davis and Domm (33) extended Goodale's work and confirmed that bilaterally ovariectomized hens do not squat for the male. In addition, ovariectomized laughing gulls

do not show any sexual behavior (34). Obviously, removal of source of gonadal hormones abolishes or weakens the sexual activities though the degree of effect may depend to some extent on age at the time of operation.

(b) Effects of gonadal hormone administration

The males.

Androgen treatment induces male copulatory behavior in the capon as has been reported by Davis and Domm (35). Further work of Domm (36) showed that capons given with testosterone propionate exhibited the normal courtship and mating pattern including crowing, "tidbitting", waltzing and treading, and achieved copulation with the female. Crowing occured within 48 hours after the beginning of hormone treatment and treading appeared within 90 hours. In addition, Domm, Davis and Blivaiss (39) observed waltzing, cirling and treading in adult capons after treatment with testosterone plus estrogen or stibestrol. However, injection of estrogen generally eliminated crowing and other forms of sexual activity in cockerels.

Further, Beach (38) reported that administration of testosterone propionate was able to revive courtship and mating in male pigeons which had lost sexual behavior following brain injury. He also showed that a sexually inactive Canada gander exhibited intense courtship and mating behavior following injections of testosterone propionate. Phillips (39) obtained courtship displays in

downy male Pintail ducklings (Anas acuta), downy Redhead ducklings (Aythya americana), downy male mallard ducklings (Anas platynhynchos) and castrated adult male mallards by daily injections of testosterone.

Results from experiments on wild birds are also consistent with the evidence which have been outlined above. Noble and Wurm stated that testosterone propionate induced in male gonadectomized laughing gulls (Larus atricilla) those breeding calls and postures common to both sexes and also those characteristics of the breeding male (34). In Herring gulls (L. argentatus) testosterone injections induced interest in territorial defense, nest building, adult male voice and courtship behavior in immature males and young male castrates (20). In another study, Noble and Wurm found that testosterone propionate induced territory defense, nesting building, courtship ceremonies, copulation and brooding in month-old chicks, in immature birds of both sexes, and in adult females of black-crowned night herons (Nycticorax n. hoactli) (40, 18). Emlen and Lorenz implanted pellets of crystalline testosterone and stibestrol in wild Valley quail of both sexes during the non breeding season and induced pursuing and then pair formation. Two testosterone treated males upon losing their mates assumed the crowing behavior typical of unpaired male during the breeding season (19) while the stilbestrol treated male did not show such behavioral re-

sponse. Apparently, various lines of evidence outlined above show that the sex behavior of male birds is strongly affected or is induced by testicular hormones.

The females.

As stated above ovariectomized females are usually sexually inactive. However, administration of ovarian hormones or related compounds can induce a return of mating behavior. Allee and Collias (7) reported that in some poulards (ovariectomized young hens) injections of estrogen restored receptive behavior. These birds were known to have previously avoided the solicitations of sexually active males. In confirmation Davis and Domm (33, 35) showed that administration of estrogen induced bilaterally ovariectomized poulards to squat and receive the courting males. Noble and Wurm (34) observed that administration of estrogen to gonadectomized female laughing gulls evoked typical female sexual responsiveness including food begging and the assumption of a stooping posture. In addition, Noble and Zitrin (13) were successful in inducing young female fowls to squat for treading males after 18 to 26 injections of estradiol benzoate, starting on the 15th day of age. These data clearly reveal the view that ovarian hormones play an important role in the control of female sexual behavior (41).

(c) Reversal of sex behavior accompanying spontaneous alteration of the gonads or following administration of hormones.

From the evidence cited it may be concluded that the

patterns of sex behavior typical of male birds in breeding conditions are directly dependent on the presence of adequate quantities of testicular androgens and similarly, although it has not been proved quite so extensively, that the female type of sex behavior is dependent on the presence of the ovarian estrogens. Changes induced in the behavior of a genetically female bird which is subjected to the influence of male hormone, and also the effects on the genetic male of the female hormones further illustrate this concept.

Many naturally occuring cases of sexual reversal have been reported (42). Brandt (43) has observed hens which showed masculine behavior in crowing, calling other hens to food, and mounting to attempt copulation. These abnormalities were found to be a result of the unusual presence of seminiferous tubules in the rudementary right gonad. Crew (42) described a series of domestic fowls which showed various degrees of sex reversal to the male type, and in some, full male behavior was recorded, the birds starting to crow and copulating with other hens. One of these hens successfully fertilized eggs from a normal female as a result of infection of the ovary and the hypertrophy of the rudimentary gonad (44). Riddle (45) reported that a ring dove originally laying eggs, later ceased laying and began to display masculine sex behavior dut to development of two testes.

Many cases of sexual reversal have been experimentally induced. In 1929, Domm (46) successfully carried out experiment of testes implantation into ovariectomized hens and found that these hens developed and retained male secondary sexual characters and behavior. The converse experiments, attempts to induce the feminization of castrated males have been less successful, and induced female characters did not last long (47). The difficulties encountered in these transplantation experiments were the development of testes tissue which exerted a male influence within the ovarian transplant (48). These difficulties were avoided in the later experiments with sex hormone injections.

Hamilton (12, 14) using testosterone propionate induced male traits, such as crowing, fighting and courtship behavior in newly-hatched female chicks and in mature hens. Also, Noble and Wurm (40) showed that testosterone propionate caused the appearance of male sexual behavior in non-breeding adult females of black-crowned night herons. Shoemaker (16) confirmed the results by injecting testosterone propionate into female canaries caused a complete suppression of female behavior and a strong development of such typical male traits as song, posturing and courtship behavior. The treated female also paired with an untreated female although attempted copulation was not seen. In addition, ordinarily passive

ring doves (Streptopelia risoria) became masculinized in courtship behavior and copulatory pattern under the influence of testosterone (17), and so did the female free-living valley quails implanted with crystalline androgen (19). Domm, Davis & Blivaise (35, 37) observed circling, waltzing and crowing in pullets and poulards treated with testosterone. Zitrin (49) successfully obtained male copulatory behavior in a hen following administration of testosterone propionate. Domm and Blivaiss (36) obtained a treading in brown Leghorn pullet implanted with pellets of testosterone propionate. The converse experiments, the injections of female sex hormone into normal adult males did not produce marked behavior changes in laughing gulls, Valley quails and canaries (34, 19, 16).

(d) Effects of hormones other than gonadal hormones.

Precocious sexual activity in young male domestic fowls has been induced after implantation of pituitaries (50, 51). Injections of hebin, a purified gonad stimulating extract of the anterior pituitaries, to young male fowls resulted in crowing and some degrees of the behavior that leads to copulation (52). Crowing occured in young males only 9 days old after six daily injection of hebin, and the first attempt of copulation was seen at the age of 13 days (53). However, similar experiments performed with young females caused hypertrophy of the ovaries without affecting the behavior (53) In these experiments, the

effects on sex behavior are believed to be caused indirectly through the stimulation of gonadal hormone secretion.

The thyroid has received much attention, but evidence of its influence on sex behavior is still inconclusive. According to Collias (54) administration of small amounts of thyroxin tend to depress the mating behavior of domestic hens and larger doses produce much more marked loss in sexual behavior which is probably related to the accompanying suppression of the ovaries. Thyroidectomized male and female brown Leghorns of eight months old showed testicular regression and reduction of egg-laying respectively (55). Feeding of thyroid substance restored the normal function in both sexes. Blivaiss (56, 57) also reported that the degree of sexual behavior was reduced in thyroidectomized brown Leghorn hens and rooster. However, it has been reported that there was no evidence of infertility when thyroidectomized hens and roosters were mated (58).

From these data it is apparent that reproductive process including sex behavior in the male and female birds require a functioning thyroid gland and that deviation in either direction may be followed by changes in, if not the elimination of, sexual behavior. However, male and female hormones directly regulate the appearance of sex behavior in males and females.

B. Hormones and Plumage in Birds.

The plumage sex dimorphism of many birds is familiar

to most of the people. Physiologists have investigated the types of control mechanism of this secondary sex character in a number of species. On the whole we can distinguish perennial and seasonal plumage changes. Among the perennial type are the species, such as pigeon, guinea fowl, Brewer's blackbird, south American plover, English sparrow and starling, with full genic control extending over both eclipse and nuptial seasons. The occasional slight seasonal changes in this group are only secondary without involving a molt being achieved but by the discarding of buff edges or spots of certain feathers by wear. In certain birds such as pheasants, the sex difference in plumages is basically determined by the sex chromosomal constitution, but is partly modified by hormones.

On the other hand, the seasonal change plumages are entirely hormone-determined. From the available information, two types of hormonal control of plumage can be distinguished. One is controlled by gonadal hormones and so reflects the gonadal activity. The other type depends on the pituitary gonadotrophin which is also in association with the seasonal gonadal activity.

1. Gonadal steroid hormone control of plumage.

(a) Domestic fowl.

This is the best known example which shows a marked sex dimorphism in the plumage. The earliest scientific study on this character was reported by Goodale (30).

After complete ovariectomy short feathers became long; straight feathers curved; feathers with broad rounded ends became narrow and pointed; salmon feathers became black; stippled brown feathers became golden with a black central stripe. By five to six months, these individuals became nearly complete replicas of the males with a brilliant, luxuriant adult male plumage. The feathers of the capons were altered comparatively little. However, daily treatment with 60 rat units of estrogens over three weeks caused hen feathering in a capon, androgen injections in capons on the other hand had no effect on plumage (59).

Compensatory growth of the right ovarian rudiment and sometimes also of remnants of the left ovary caused an initial cock feathering followed by reassumption of hen plumage in poulards (41). After removal of these gonadal regenerates, the poulards resumed cock plumage again, showing no subsequent reversion to female plumage as did the females in which successful bilaterally ovariectomy had been achieved (60). Moreover, implantation of ovaries feminized the plumage in accordance with the degree of regression of the graft (61). All these experimental results led investigators to explain that the cock plumage is a neutral form whereas the hen plumage is estrogen controlled. Another possible explanation is that the chicken pituitary has the tendency of releasing gonadotrophin LH throughout the year in sufficient amounts to produce cock

feathering, the hen plumage forms only whenever estrogens suppress the LH output. Consequently, complete ovariectomy, removing the source of estrogens, creates an unopposed LH condition which would result in cock feathering. Although a gonadotrophic mechanism could explain the facts, there is still lack of experimental evidence that it is involved. The observed facts can also be explained on the assumption that the male plumage is genetically determined but suppressed by estrogens in normal females.

(b) Ducks.

The female Rouen ducks wear a hen plumage which is comparatively little changed during the breeding season whereas the males put on a summer or eclipse plumage at the height of the breeding season, which is retained for 3 or 4 months. Early in the fall both sexes molt but only the males resume a nuptial cock plumage at a time the testes are in regression. Ovariectomized Rouen ducks assumed a permanent male plumage similar to that of castrated drakes. Castrated drakes did not develop the summer or eclipse plumage of the normal males (30). This suggests that the cock plumage in Rouen duck is neutral and the hen plumage is estrogen controlled and the male summer or eclipse plumage is gonad dependent.

Similarly, a female mallard duck wears a hen-type plumage except for a bright white and blue speculum which is a feature of the cock plumage. The male assumes the

cock plumage after the post nuptial molt (September), when the testes have already regressed. At the height of breeding season, the male molts and assumes the male summer or eclipse plumage for three months. Castrations of male and female mallards cause permanent assumption of cock plumage. By systematic plucking and registry of the regenerating feathers it has been found that the eclipse plumage is hormonally induced by the testes in full activity (61). Thus at the height of breeding season the mallard drake is in the eclipse plumage. However, Caridroit (62) reported that estrogens but not androgens could induce the eclipse plumage in castrates.

In addition, if drakes are castrated close to the breeding season, they first resume cock feather followed by a transitory period of hen feathering which is somewhat delayed in development in comparison with that of intact male. In the following years the same male permanently produces cock feathers (61). Above all the sexual plumage in this species appears to be gonadal steroid controlled but the experiments need to be extended to complete the analysis.

(c) Ruff, Blackheaded gull, Herring gull and phalarope.

The ruff wears a dull coloured hen like plumage outside the breeding season. After the spring molt, the male dons a colorful nuptial plumage with the "ruff" of long pectoral feathers. Castration in early winter prevents

the development of the male nuptial plumage (68). This suggests that androgens control the male nuptial plumage. The female also assumes a rudimentary male nuptial plumage though no ruff is formed. Since in birds the secretion of male hormone by normal ovaries has been demonstrated clearly in the work of Witschi and Miller (63) the rudimentary cock-type plumage of female is not inconsistent with the suggestion of androgen control of cock plumage in ruff.

Similarly, blackheaded gulls have a dark head during the breeding season, in both sexes, which changes to white after the fall molt. Castration of the male prevents the assumption of cock plumage (61). These observations also suggest the androgen control of cock plumage in blackheaded gull.

Direct evidence of androgen control of plumage has been obtained from the studies on the Herring gull. In this species, the adults exhibit neither a sexual nor a marked seasonal plumage dimorphism. But the young wears a hen-type juvenal plumage for three years, which is not altered either by castration or by continuous injections of estrogen. A precocious development of the characteristic white and silver-gray adult plumage is induced by administration of androgen (20), though whether or not castration permanently prevents the development of adult plumage is still unknown.

In phalaropes, the subject of our present study, the female wears a brighter nuptial plumage than the male and both sexes have a similar dull fall and winter plumage. As has been noted above that the growth of the colorful nuptial plumage is correlated with the onset of aggressive courtship behavior in female in the beginning of the breeding season. Because of the apparent association of aggressiveness and the nuptial plumage in female it was suspected that androgenic hormones produced by the ovary might control the development of the colorful female nuptial plumage. While we were carrying out steroid hormone analysis on phalarope gonads, Johns (64) obtained experimental results which showed that testosterone but not estrogen given to birds in the fall or winter plumage induced the growth of brilliant nuptial plumage feathers in Wilson's and Northern phalaropes of either sex. Our finding that ovaries of breeding Wilson's phalaropes do in fact contain more testosterone per unit body weight than the testes of the conspecific males provides a piece of indirect evidence for androgen control of nuptial plumage in phalaropes

(d) Hen-feathered chickens.

After castration, both male and female of purebred hen-feathered Sebright bantams put on a cock plumage (65). Injections of either estrogens or androgens reduce the effect back to hen feathering (66). Danforth (67) has shown that skin patches of hen-feathered males or females

transplanted on a cock-feathered male still produce hen feathers; conversely, skin of cock-feathered males or females transplanted on a hen-feathered male regenerate cock feathers. This indicates that the genetic difference determines the threshold value of feather germs to react to the androgen levels of cock in this case, since independent of genetic sex the feather germs of mutant gene stock produce only hen feathers while under identical conditions feather germs of normal stock develop into cock feathers and the androgen levels in males of these two types of breeds have been shown to be essentially the same (68).

Moreover, plumage of pheasants hold an intermediary position between hormonal and genetic control types. After exchanging skin grafts between males and females, the transplants grow feathers of two new intermediary types (69). Estrogens as well as androgens can partially feminize the feather pattern (61). Apparently, the sex dimorphism in pheasant plumages is basically genetically controlled but is modified by the gonadal steroid hormones.

2. Gonadotrophic control of plumage.

Weaver finches have been clearly shown to exemplify the gonadotrophic control mechanism of plumage. The orange and yellow weaver finch females constantly wear a typical hen plumage of white breast feathers which is molted once a year. The males change seasonally between a nuptial cock plumage of black and orange-red and the eclipse hen

like plumage. This requires a double molt. Castration does not change the cycle of plumage changes of the males. The same cock-type periodicity in plumage changes is induced in ovariectomized females (70). This implys that the feather pigmentation which occurs during the male phase of the plumage cycle is not controlled by gonadal androgens and that in normal female development of cock plumage is prevented by the early release of ovarian estrogens. The latter point was confirmed by estradiol and other gynegenic steroid injections which prevented the differentiation of cock plumage in males at the time they would if untreated be assuming the cock plumage of the breeding season. Androgens in large dosages also suppressed the differentiation of cock plumage. However, simultaneous injections of gonadotrophin with androgens still resulted in typical nuptial plumage (61). Obviously, these effects are not directly due to androgen and it is concluded that gonadotrophin induces cock plumage in this group of birds.

To determine the particular gonadotrophin that controls the development of cock plumage, Witschi (71) showed that even one rat unit of pregnant mare serum which contained FSH, ICSH and LH, was effective in inducing cock feathering. Injection of hypophysial suspensions into any bird, quiescent male or female, castrated or intact, induced the deposition of pigment in regenerating feathers and so did the injections of human chorionic gonadotrophin (72). It

is evident that FSH and ICSH are present in both sexes throughout the breeding season and both singly or in combination injections of these hormones do not induce the cock plumage (73). Therefore, it is quite clear that in the weaver the cock plumage is controlled by LH and the hen plumage appears to be neutral. A similar control mechanism of the plumage has also been shown in the American indigo bunting (Passerina cyanea), the masked weaver or dioch (Quelea quelea) and the paradise wydah (Steganura paradisea). However, it has been shown that androgens help the assumption of regular molt and replacement of a good plumage due to its mobilizing effect on the carotenoid store in the liver. Thus the new plumage is more or less brightly dyed orange and red. Androgens thus still play some role in the pigmentation of plumage in the group of birds which are shown to have an LH controlled nuptial plumage.

III. Materials and Methods:

A. Gonads.

Since 1962, Wilson's phalaropes were collected from the time of spring arrival in May to mid July, within 25 miles of Edmonton, Alberta. The birds were shot in the field and were brought to the laboratory in a plastic bag with dry ice. The gonads of both sexes were dissected out and weighed. They were then kept frozen in normal saline until a batch for the whole season had been ob-

tained. In 1963 and 1964 frozen phalarope gonads were also collected for us by Dr. E. W. Pfeiffer of Montana State University from the Missoula district of Montana, U.S.A.

In 1963, both ovaries and testes were divided into two batches representing the growing and declining gonads, i.e. those collected from the time of arrival until the maximal gonadal weights were reached and those collected thereafter. In 1964, the phalarope ovaries only were divided into three batches representing the pre-laying, laying and post-laying periods, according to the appearance of the ovaries and the diameters of the follicles at the time the ovaries were collected. The 1964 phalarope testes were combined into one batch.

For comparison, gonads of male and female mallard ducks (Anas platyrhynchos), domestic ducks, domestic fowl (Gallus **gallus**), and those of domestic pigeons (Columba livia) were also obtained. In addition, gonads of female and male Redwinged black birds (Agelaius phoeniceus) were collected from early May to late July in 1964, and in 1962, gonads of Killdeer plovers (Oxyechus vociferus) were also collected.

All the collected gonads were used for extraction and paper chromatographic analysis of gonadal steroid hormones.

B. Preparation of extracts.

The weighed gonads were homogenized with 0.9% NaCl in

a waring blender or with pestle and mortar if the tissue was very small. Twice distilled acetone double the volume of the homogenate was added for the precipitation of the proteins. The sample was allowed to stand overnight in a refrigerator. It was then filtered through a Buchner funnel with Whatman #1 filter paper. The flask and the protein precipitate were washed three times with an acetone water mixture (2:1) of 10 mls each time. The combined filtrate was concentrated in the flash evaporator, under reduced pressure, until all acetone was removed.

Distilled methanol was then added to the remaining aqueous filtrate to make up a 70% methanol mixture. This mixture was kept in the freezer overnight for fat freezing. The frozen mixture was then filtered rapidly and the frozen fat residue was washed with ice cold 70% methanol. With samples from small amount of tissue, this fat freezing procedure was omitted. The methanol was evaporated off in a flash evaporator in vacuo at a temperature of 40°C. The aqueous solution was then extracted three times with peroxide free purified ether using 50 mls each time or more when the sample was more than 50 mls. The combined ether extract was washed successively with a concentrated carbonate buffer (PH 10.5) 30 mls, 1M sodium bicarbonate solution 10 mls and 30 mls of distilled water. The ether extract was then evapor-

ated to dryness by steam bath. The remaining aqueous phase was then re-extracted three times with distilled carbon tetrachloride using the same volume as that of ether previously used. The combined CCl_4 extract was treated with sodium sulfate to remove the water. The Na_2SO_4 was filtered off and the extract was evaporated to dryness by means of a steam bath with vacuum. The ether and carbon tetrachloride extracts were transferred and combined in a small tube with a chloroform methanol mixture (1:1). The combined extract was then evaporated in a stream of nitrogen gas with the tube in a water bath at 40°C. It was then ready for paper chromatography.

C. Paper chromatography (Fig.1).

The extract was dissolved with about 1.5 ml methanol chloroform mixture (1:1) and was applied on washed chromatographic paper impregnated with glycol methanol mixture (1:1) to chromatograph for 4 days in a Hexane propylene glycol system (74), with a parallel run of small amounts of estrone, estradiol and testosterone standards applied on the standard strip of the paper. The overflow from this paper containing progesterone and androstenedione was dried by evaporation and the residue was dissolved and applied on a second Hexane propylene glycol solvent front system with a blank strip and a standard strip to which androstenedione and progesterone standards were applied. From this chromatogram androstenedione and progesterone

were eluted with distilled methanol. If the eluates were not pure, they were rechromatographed with corresponding standards and blank strips to get pure eluates from each zone.

The eluate from the testosterone zone of the original paper was evaporated to dryness and was acetylated with acetic anhydride and pyridine. After acetylation, the testosterone extract was applied to a solvent front Hexane propylene glycol paper with standards of testosterone acetate and 17-OH-progesterone on a standard strip along with a blank strip. The testosterone acetate zone and the corresponding blank zone were eluted with methanol. The positions of progesterone, androstenedione, testosterone acetate and 17-OH-progesterone were determined by means of ultraviolet light absorption of each standard. These purified chromatograms were eluted with methanol and were evaporated to complete dryness for the Isonicotinic acid hydrazide (INH) color reaction.

The eluate of the estrone zone of the original paper was applied to a dry chromatographic paper with estrone and estradiol standards. The paper was equilibrated dry in a Heptane methanol system for 1 hour before the mobile phase was added. This paper was run for 40 hours. The estradiol zone including the origin of the paper was eluted and was chromatographed either in a Heptane methanol system for 64 hours or in a Chloroform formamide solvent

front paper , with estrone, estradiol and estriol standards. Each estrogen zone was located by the Berlin blue reaction (75). The eluates from the estrone and estradiol zones from the two papers were combined. The combined eluate was then rechromatographed for purification on Heptane methanol paper for 40 hours or on hexane propylene glycol for 5 days. The estradiol zone was applied on a chloroform formamide solvent front paper impregnated with acetone formamide (11:5). The eluate of the estriol zone from the 40 hour Heptane methanol paper was purified by chromatography in the solvent front benzene butanol, methanol water system of Ulstrom system IV (76). All the purified chromatograms of estrogens were eluted with redistilled ethanol and they were evaporated to complete dryness for the Folin color reaction (77).

D. Colorimetric determination of purified steroid hormones.

1. Folin color reaction.

The dry eluates from the estriol, estradiol and estrone zones as well as their corresponding blank zones were lined up. The residues were dissolved in 0.06 ml purified ethanol. 10, 20, and 30 g of each estrogen standard and reagent blank were set up in the same way. To each sample 3 ml. Folin reagent (commercial Fisher Folin phenol reagent, 1 ml. diluted with 40 ml. distilled water) was added and allowed to stand at room temperature for 30 minutes in the dark. A blue color developed and

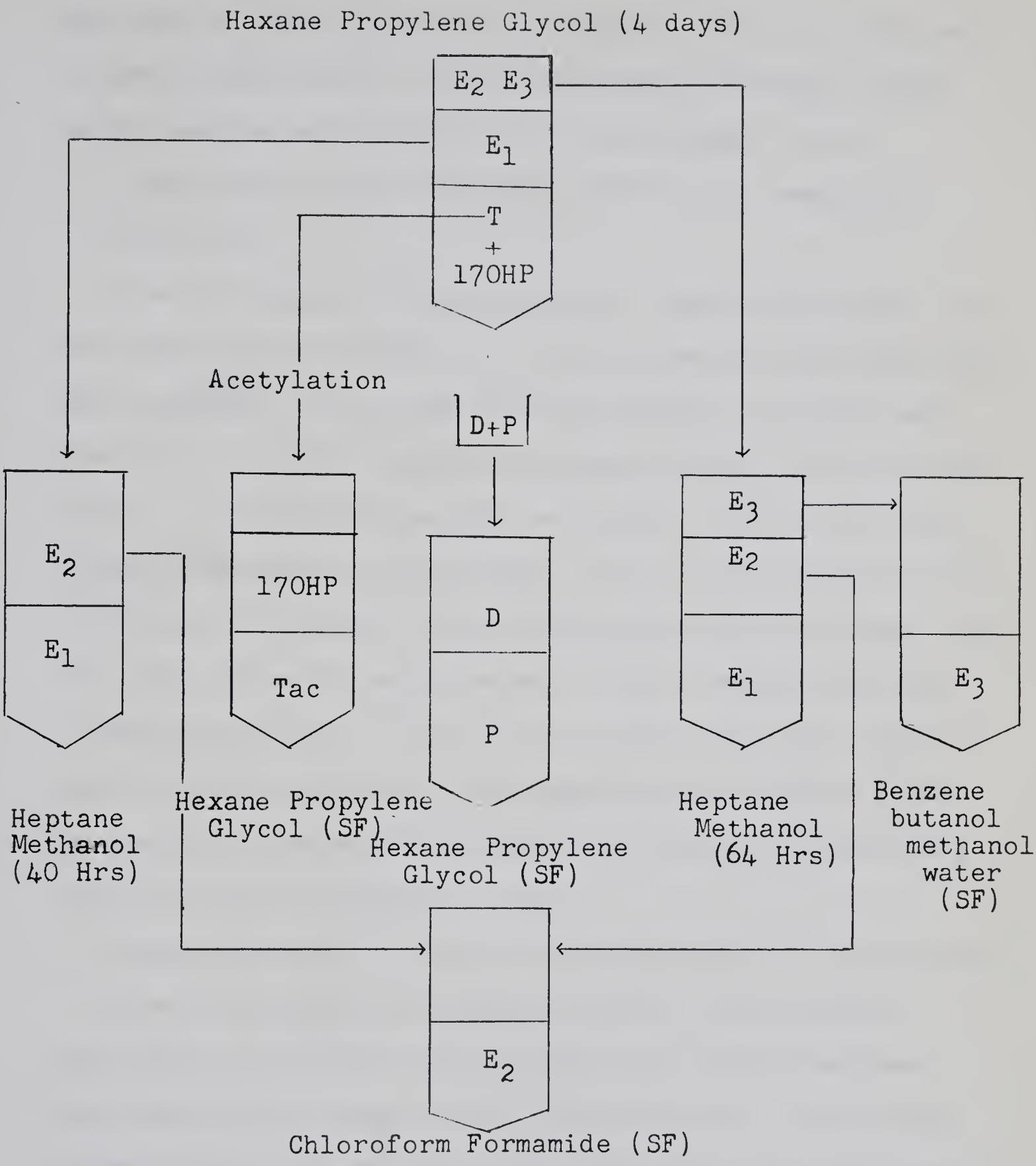

Figure I. Scheme of chromatographic procedure used for the separation of steroid from gonadal extracts.

E_1 : Estrone ; E_2 : Estradiol ; E_3 : Estriol.

P : Progesterone ; 17OHP : 17-OH-Progesterone;

T : Testosterone ; T : Testosterone acetate; D : Androstenedione.

was read at 760 mμ against the reagent blank in a Beckman Du Spectrophotometer. The micrograms of estrogen found in the sample was obtained from the standard curve.

2. Isonicotinic acid hydrazide (INH) color reaction (78, 79).

The dry eluates of progesterone, androstenedione, testosterone acetate as well as their corresponding blank zones were lined up. 25 mg INH (Taylor Chemical Company) was dissolved in 100 ml acidified ethanol (0.625 ml concentrated HCl to 1000 ml ketone free ethanol). Three ml of this reagent was added to each tube. 10, 20, and 30 μg of each standard and a reagent blank were prepared in the same way. The tubes were allowed to stand at room temperature for 1 hour in the dark. A yellow color was developed and was read in quartz cells at 370 mμ against the reagent blank. The amounts of steroid hormones found in the samples were obtained from the standard curve.

E. Identification of the steroids measured in the extracts.

From an extract of breeding domestic duck testes, testosterone acetate, progesterone and androstenedione were obtained by means of the chromatographic method described above. The samples were identified with the standards by their sulphuric acid chromogen spectra (80, 81).

The fact that an estrogen was present in the estradiol and estrone chromatographic fraction obtained from the same duck testes was demonstrated by the effect of subcu-

taneous injections of these fractions on the vaginal smears obtained from spayed mice.

F. Assays of prolactin contend in pituitaries.

1. Bio-assay.

Female and male Wilson's phalarope or Killdeer pituitaries were implanted subcutaneously over the crop sacs of the common domestic pigeons which were known to have inactive crop sacs at the start of the tests. by the technique described by Bailey (8), or they were injected intradermally (82, 83) over the crop sac in saline or water extract suspension. For controls piece of phalarope brain or 10 ug prolactin was implanted or injected over the other side of the crop sac. After 4 days the pigeons were killed. The crop sacs were removed and examined macroscopically for proliferation of the epithelium and then they were fixed in 10% formalin, sectioned, and stained by Lillies' azure A - eosin B technique (84) which differentiates the prolactin response of the crop sac epithelium from mere thickening due to non-specific irritants (85).

2. Cytological study.

Both male and female pituitaries of Northern phalaropes were fixed in Zenker formol. They were then sectioned at 5u thick and stained by the method described by Höhn (86). The acidophils being stained orange-red were believed to be prolactin cells. They were counted in 45 unit areas (about 0.06 mm^2, representing about 2% of the area of the whole

section). Five different sections of each pituitary were counted. The means of acidophil counts in both sexes were tested for significant difference by the method of t test.

3. Indirect evidence from pituitary weights.

The weights of pituitaries from the breeding season Wilson's phalaropes, Redwinged black birds and Killdeers were recorded. They were analyzed statistically for the difference in both sexes.

IV. Results:

A. The contents of gonadal steroids in phalaropes and other birds.

The results obtained from the paper chromatographic analysis and colorimetric determination are summarized in tables I and II. The gonadal steroids other than progesterone which is probably not significantly related to the present study, per unit body weight obtained from various gland samples are shown graphically in figure 2 and 3.

It was found that concentration of steroid hormones per unit gonad weight of post breeding mallard ducks was higher than that of breeding mallards in both sexes. This can be well accounted for by the very large proportion of tubular and non-hormone producing tissue in the breeding testes while in the non-breeding condition the proportion of hormone producing interstitial tissue is relatively

greater. In the case of females a large proportion of the weight of breeding season ovaries is made up by the yolk of the enlarged yolk filled follicles so that the proportion of hormone producing tissue is less in the breeding than that in the non-breeding state.

When the gonadal steroids were expressed per 100 gm body weight, so as to make the figures for the different species comparable, it was found that the effective gonadal estrogens as well as androgens were greater in amount in breeding than in non-breeding mallard gonads of both sexes (fig. 2, 3). In addition, there was a progressive decline in the values of androgens and estrogens per 100 gm body weight during the course of the breeding season in the case of both male and female mallards. This indicates that in a general way, gonadal steroid concentrarions reflect the amount of hormones secreted.

For Wilson's phalaropes it was found that phalarope ovaries were unusually rich in testosterone when compared to all the other birds examined except the domestic pigeon and Killdeers. Firstly, the ovaries of four out of six of the phalarope samples contained more testosterone per unit body weight than those of the testes of conspecific males collected during the corresponding period. Secondly, the ratio of ovarian testosterone/estradiol of 0.6/1.0 in three out of five samples, considerably exceeded this ratio for all the other species examined of 0.1/1.0 - 0.5/1.0, except

the Killdeers in which the ovaries appeared to contain more testosterone than estradiol. The ovarian testosterone/estradiol ratio of phalarope markedly exceeded the ratio found in the ovaries of mallards and domestic fowls which are known to have the most obvious dominant males when compared to the other species examined. In the case of the domestic pigeon, although the ovaries contained more testosterone than the testes of the conspecific males, there was more estradiol relative to testosterone in the ovaries than in the majority of the phalarope ovary samples. The ratio of ovarian testosterone/estradiol of the domestic pigeons was 0.5/1.0 while that of the majority of phalarope was 0.6/1.0 (table I).

In comparing the testosterone content of breeding mallards and domestic fowl testes with that of phalaropes, there was a considerably lower testosterone content in Wilson's phalarope testes even when only the growing gonads of sample I for 1963 were considered. However, phalarope testes were not greatly deficient in testosterone when compared to those of male Redwinged blackbirds. Their testosterone/estradiol ratio was somewhat low in comparison with that found in the testes of other species examined. The results also indicated that male phalaropes are quite good estrogen producers.

From the results of Sulphuric acid chromogen spectra, the extracted testosterone showed the identical spectrum

Table 1: Gonadal Steroid in the Ovaries of Wilson's Phalaropes and certain other birds. n.m.=not measured, n.f. - none found.
In each case the first column records micrograms per g. of tissue, the second micrograms per 100 g. body wt.

Species etc.	Ws.Phal.1962		Ws.Phal.1963 I		Ws.Phal.1963 II		Ws.Phal.1964					
No. of birds in sample	9		26		32		prelaying 13		laying 11		post laying 6	
Wt. of tissue extracted	0.93g		10.11g		10.08g		1.88g		5.47g		1.95g	
Testosterone	1.61	0.25	0.15	0.09	0.96	0.46	1.41	0.29	0.95	0.70	1.56	0.76
Androstenedione	5.38	0.87	1.14	0.68	3.36	1.62	2.59	0.53	2.11	1.56	1.68	0.83
Progesterone	1.61	0.25	0.95	0.56	0.70	0.33	2.24	0.46	0.99	0.73	0.98	0.48
Oestrone	2.70	0.41	1.49	0.88	1.02	0.49	0.35	0.07	0.27	0.20	0.71	0.35
Oestradiol	n.f.	n.f.	2.51	1.49	2.45	1.18	2.44	0.50	1.64	1.22	2.61	1.28
Oestriol	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	0.13	0.09	0.63	0.42	1.36	0.67
Ratio $\frac{\text{Testosterone}}{\text{Oestradiol}}$			$\frac{0.06}{1}$		$\frac{0.4}{1}$		$\frac{0.6}{1}$		$\frac{0.6}{1}$		$\frac{0.6}{1}$	

Table 1 - Continued

Species etc.	Red-wd.Bl.bird 1964		Dom.Pigeon breeding		Dom.Hen laying		Mallard 1963 breeding		Mallard 1964 I		Mallard 1964 II		Mallard 1964 III		Mallard 1964 IV pest breeding	
No. of birds in sample	24		2		1		2		1, May 18		1, May 23		1, May 25		3	
Wt. of tissue extracted	2.87g		1.49g		14.90g		26.63g		41.1g		2.44g		1.40g		0.65g	
Testosterone	0.59	0.14	4.61	1.11	0.32	0.19	0.36	0.40	0.08	0.29	n.f.	n.f.	1.72	0.25	6.33	0.14
Androstenedione	1.19	0.28	1.57	0.38	0.84	0.50	0.37	0.42	1.60	5.85	1.89	0.43	0.99	0.14	6.50	0.15
Progesterone	0.63	0.15	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	1.30	4.76	2.00	0.45	1.73	0.25	4.70	0.11
Oestrone	1.35	0.32	3.29	0.79	n.f.	n.f.	0.84	0.93	0.23	0.83	2.03	0.46	n.f.	n.f.	3.11	0.07
Oestradiol	4.91	1.15	8.81	2.12	2.52	1.5	1.37	1.52	0.74	2.70	4.15	0.94	6.79	0.98	19.8	0.46
Oestriol	1.25	0.29	0.13	0.03	n.m.	n.m.	n.m.	n.m.	0.15	0.56	0.04	0.01	2.46	0.35	5.05	0.12
Ratio $\frac{\text{Testosterone}}{\text{Oestradiol}}$	$\frac{0.1}{1}$			$\frac{0.5}{1}$	$\frac{0.1}{1}$		$\frac{0.26}{1}$		$\frac{0.1}{1}$				$\frac{0.25}{1}$		$\frac{0.30}{1}$	

Table 1 - Continued

Species etc.	Killdeer 1962	
No. of birds in sample	21	
Wt. of tissue extracted	4.74g	
Testosterone	1.22	0.27
Androstenedione	1.51	0.34
Progesterone	0.53	0.12
Oestrone	2.09	0.47
Oestradiol	0.10	0.02
Oestriol	n.m.	n.m.
Ratio $\frac{\text{Testosterone}}{\text{Oestradiol}}$		$\frac{13.5}{1}$

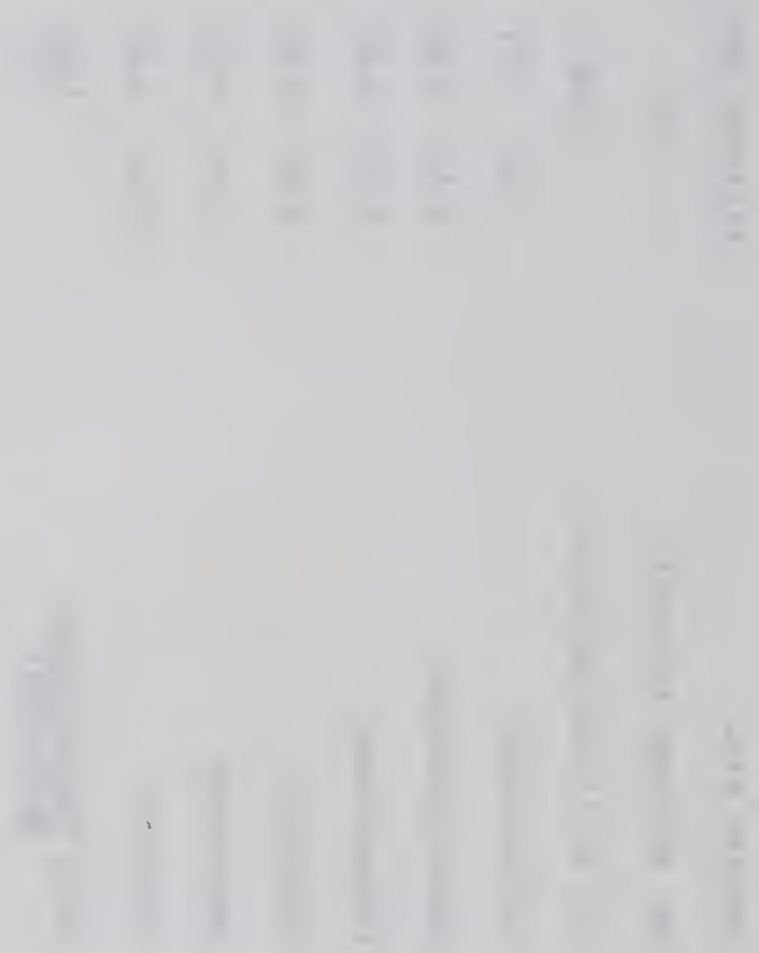

Table 2: Gonadal Steroids in the Testes of Wilson's Phalaropes and certain other birds. In each case the first column represents micrograms per g. of tissue, the second, micrograms per 100 g. body wt.
n.m. = not measured, n.f. - not found.

Species etc.	Ws.Phal.1962		Ws.Phal.1963 I		Ws.Phal.1963 II		Ws.Phal.1964	
No. of birds in sample	45		15		55		19	
Wt. of tissue extracted	2.35g		4.15g		7.45g		4.46g	
Testosterone	0.43	0.04	0.72	0.38	1.26	0.33	1.23	0.56
Androstenedione	3.96	0.41	3.98	2.14	2.37	0.62	0.64	0.29
Progesterone	1.28	0.13	1.63	0.87	0.85	0.22	0.20	0.09
Oestrone	2.24	0.23	0.41	0.22	1.16	0.31	0.25	0.11
Oestradiol	n.f.	n.f.	2.07	1.11	2.07	0.55	3.22	1.51
Oestrial	n.m.	n.m.	n.m.	n.m.	1.05	0.27	1.62	0.74
Ratio $\frac{\text{Testesterone}}{\text{Oestradiol}}$				$\frac{0.36}{1}$		$\frac{0.6}{1}$		$\frac{0.4}{1}$

Table 2 - continued

Species etc.	1964 Red-wd.Blackbirds breeding		1964 Dom.Pigeon breeding		1963 Cockerel breeding	
No. of birds sampled	23		5		1	
Wt. of tissue extracted	12.02g		12.23g		28.8g	
Testosterone	0.87	0.60	0.85	0.61	7.38	9.6
Androstenedione	0.37	0.25	0.74	0.52	0.25	0.33
Progesterone	0.96	0.65	0.38	0.27	0.16	0.20
Oestrone	0.02	0.02	0.39	0.28	0.6	0.76
Oestradiel	0.93	0.64	0.65	0.47	0.9	1.13
Oestriel	n.m.	n.m.	0.55	0.40	0.3	0.40
Ratio $\frac{\text{Testesterone}}{\text{Oestradiel}}$		$\frac{0.9}{1}$		$\frac{1.3}{1}$		$\frac{8.3}{1}$

Table 2 - continued

Species etc.	Mallard 1962 breeding		Mallard 1962 post breeding		May 18 I		Mallard 1964 breeding May 25 II		June II III		Killdeer 1962	
No. of birds sampled	1		2		1		1		1		27	
Wt. of tissue extracted	12.8g		0.291g		19.16g		12.23g		13.17g		0.94g	
Testosterone	3.85	4.10	4.14	0.1	1.5	2.3	0.98	1.07	0.51	0.56	9.5	0.36
Androstenedione	2.10	2.30	32.80	0.70	0.9	1.46	1.30	1.42	0.63	0.69	26.5	1.00
Progesterone	2.26	2.37	34.36	0.42	1.60	2.43	0.69	0.75	0.57	0.63	16.4	0.62
Oestrone	3.00	3.30	9.66	0.23	0.10	0.16	0.16	0.17	0.24	0.26	15.10	0.57
Oestradiol	0.05	0.05	n.f.	n.f.	0.28	0.43	1.14	1.25	0.32	0.35	27.5	1.05
Oestriol	n.m.	n.m.	n.m.	n.m.	0.28	0.44	n.m.	n.m.	0.33	0.37		
Ratio $\frac{\text{Testosterone}}{\text{Oestradiol}}$	$\frac{82}{1}$				$\frac{5}{1}$		$\frac{0.8}{1}$		$\frac{1.6}{1}$		$\frac{0.6}{1}$	

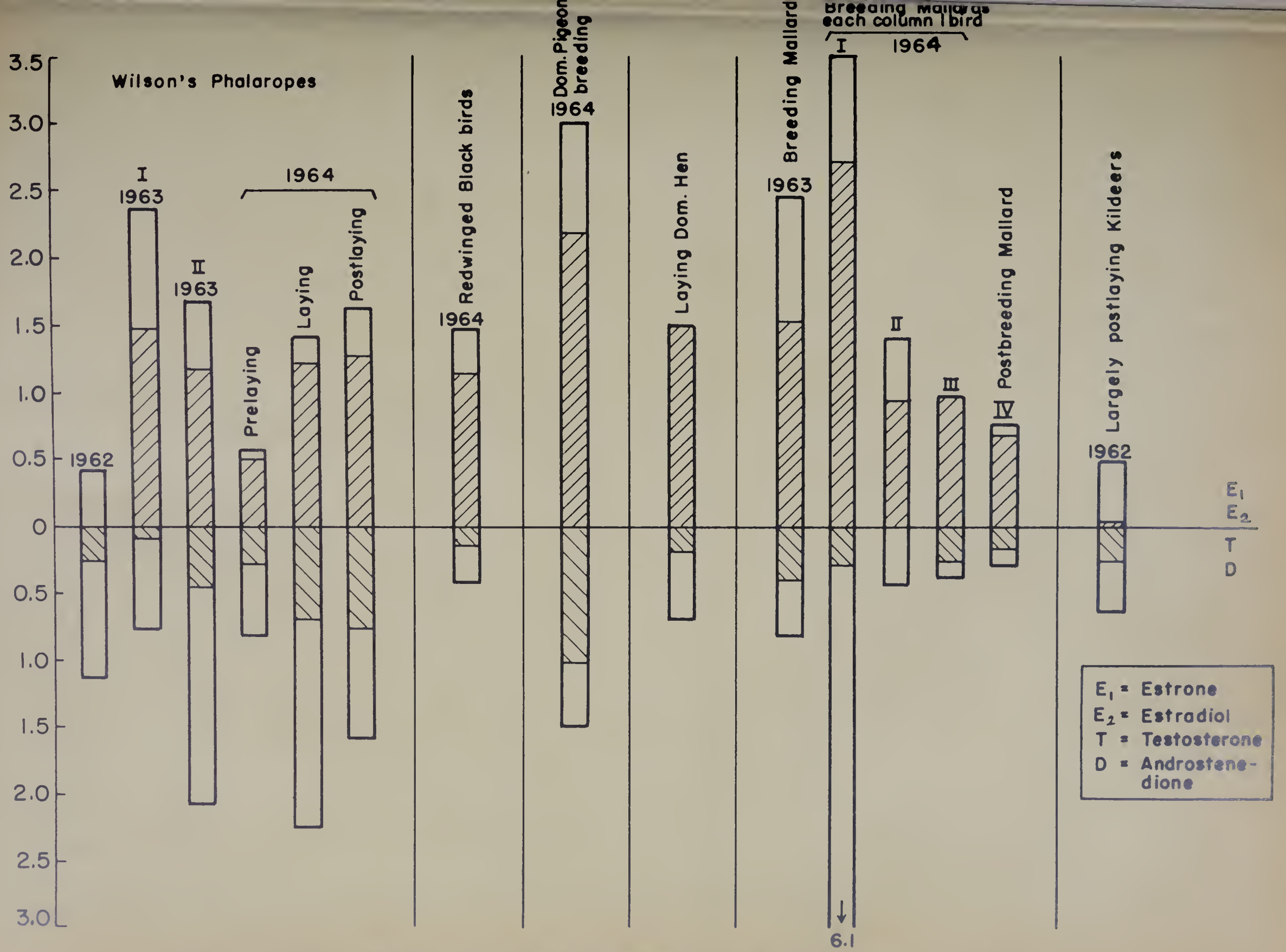

Fig. 2. Ovarian steroid Hormones expressed at µgs/100g body wt of bird. Estrogens above the line in the order E_2, E_1, Androgens below the line in the order T, D.

µg /100 g body wt. of bird

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

Wilson's Phalaropes
1962
1963
I
II
1964
Redwinged Black birds
1964
Breeding Dom. Pigeons
1965
Breeding Dom. Cock
1963
9.6
D=0.33
Breeding Mallard
1962
6.4
4.1
Breeding Mallards
1964
3.7
Postbreeding Mallard
1962
Killdeer
1962
E1
E2
T
D

with the standard testosterone. In the case of progesterone and androstenedione the spectra failed to show their identity due to insufficient purification of the samples. For estrogen extract tests, the appearance of partially cornified vaginal epithelium in the spayed mice indicated the presence of small amount of estrogens in the esterone and estradiol chromatographic fractions obtained from the breeding duck testes.

B. Prolactin content in phalarope pituitaries.

1. Pigeon crop sac tests.

The results of experiments with implants of Wilson's phalarope or Killdeer plover pituitaries over the crop sac of common domestic pigeons or by means of intradermal injections over the crop sac are shown in the table 3.

Although in no case did we obtain a full proliferition of the crop sac with up to 10 g of prolactin (ovine supplied by the Endocrinology study section U.S. Natl. Insts. of Health) or with the pituitary glands, the data showed that breeding male phalaropes produce some prolactin while females produce none or significantly less. The two tests with Killdeer plover pituitaries allow no conclusion.

2. Cytological study.

The average acidophil count per unit area (0.06 mm^2) from five different pituitary sections of each bird is tabulated in table 4. The average acidophil count of each sex is expressed graphically in figure 4. It was evident

that male Northern phalarope pituitaries have a larger number of acidophils than those of females. The means of the acidophil counts in males and females were 223±26.4 and 108±32.9, respectively. Comparison of the two means by the t test gave a t value of 2.84. The t value required for significance at the 0.05 level was 2.77. Hence the difference in acidophil counts in male and female pituitaries is significant at the 5% level.

Acidophils are believed to be prolactin as well as somatotropic hormone (STH) producing cells (87, 88, 89). Although differentiation of two types of acidophils as STH-o-cyte and prolactinocyte was not obtained in this experiment it is highly probably that most of the acidophils shown were prolactinocytes, since all the pituitaries were obtained from fully grown adult birds which are believed to have few STH cells in the pituitaries. Besides, the incubation patches were present in all the males from which the pituitaries were removed for the present study.

3. Relative pituitary weights of breeding season Wilson's phalaropes, Redwinged blackbirds and Killdeers.

The data are summarized in table 5. The unit of body weight of 50 gms relative to which the pituitary weights were expressed in the table is approximately the mean body weight of the smaller of the two sexes in Wilson's phalaropes and Redwinged blackbirds. In the Wilson's phalaropes only male while in Redwinged blackbirds only

Table III

PIGEON CROP SAC TESTS FOR PROLACTIN, USING IMPLANTS OR INTRADERMAL INJECTIONS OF SALINE OR WATER EXTRACT-SUSPENSIONS OF BIRD PITUITARIES APPLIED FOR 4 DAYS.

Designation of Test Pigeon (Year and Expt. No.)	Implant I or Injection of Extract E of Pituitary	Total Dose applied to Experimental side	Response of Experimental side	Total Dose or Test tissue to Control side	Response of Control side	Remarks
'61 3	I	15 ♂ Phal. Pits.	Mac*: - + Mic.: - +	Piece Phal. Brain	Mac: 0 Mic: 0	
'61 4	I	14 ♂ Phal. Pits.	Mac.: - + Mic.: 0→- +	Piece Phal. Brain	Mac: 0 Mic: 0	
'61 5	I	18 ♀ Phal. Pits.	Mac.: 0 Mic.: n.r.**	Piece Phal. Brain	Mac: 0 Mic: n.r.	
'62 40	E	E. of 15 ♂ Killdeer Pits.	Mac.: 0 Mic.: 0→- +	Prolactin 10γ	Mac.: + Mic.: - +	mainly post breeding Pits.
'62 67	E	E. of 15 ♀ Killdeer Pits.	Mac.: 0 Mic.: 0	Prolactin 10γ	Mac.: + Mic.: - +	
'62 48	E	E. of 39 ♂ Phal. Pits.	Mac.: + Mic.: +	Prolactin 10γ	Mac.: + Mic.: - +	
'63 2	E	E. of 20 ♂ Phal. Pits.	Mac.: 0 Mic.: n.r.	Prolactin 10γ	Mac.: - + Mic.: +	Pigeon died after only 3 daily treatments
'63 1	E	E. of 40 ♀s Phal. Pits	Mac.: 0 Mic.: 0	Prolactin 3γ	Mac.: - + Mic.: +	
'64 1	I + E	I of 17 ♂ Phal. Pits. + 3 doses of ♂ Phal. Pit. Extract	Mac.: + Mic.: n.r.	nil	Mac.: 0 Mic.: n.r.	

Footnote * Mac. = Macroscopic ** n.r. = not recorded "Scale" of crop sac responses; 0=no response at all
Mic. = Microscopic -+ = suggestive evidence of positive response
N.B. - all pigeons used were known to have inactive crop-sacs at the start of the tests + = minimal but definite positive response

Table 4

The acidophil counts in pituitaries of male and female northern phalaropes.

Bird No.	Average acidophil count per unit area ($0.06mm^2$) ± S.E.	
	Male	Female
1	202 ± 28.3	146 ± 10.4
2	195 ± 6.7	42 ± 6.5
4	272 ± 9.9	135 ± 5.6
Average	223 ± 24.6	108 ± 32.9

t value = 2.84*

S.E. = Standard error of a mean

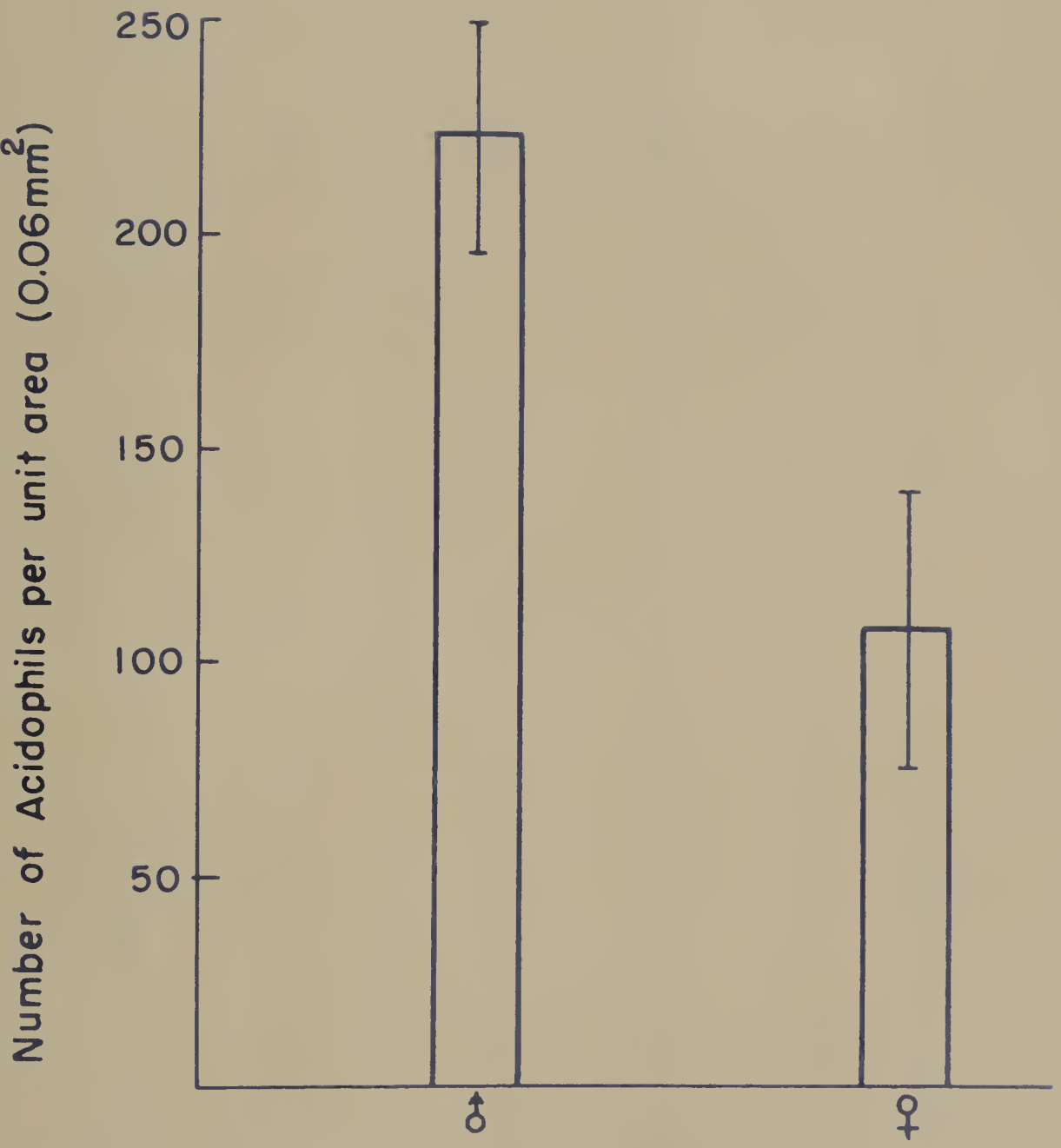

Fig. 4. Acidophil counts in pituitaries of male and female Northern Phalaropes.
Each bar represents the mean ± S.E. for 3 birds.

Table V

BODY AND PITUITARY WEIGHTS AND RELATIVE PITUITARY WEIGHTS OF BOTH SEXES IN WILSON'S PHALAROPE AND IN TWO OTHER SPECIES OF BIRDS. For each species the sex which, in nature, incubates and develops a brood patch is indicated in the last column. The years during the breeding seasons of which birds were collected are shown in brackets.

Species and Sex	Body Wt. g. Mean and st. error of mean.	Pituitary Wt. mg. Mean and st. error of mean.	No. of birds examined	Relative Pituitary Wt. (Pituitary Wt./50g. body Wt.	Natural formation of brood patch and incubation.
Wilson's Phalarope Females (1962, '63 and '64)	68.2 ± 1.8	1.40 ± 0.04	28	1.20	-
Wilson's Phalarope Males*(1962, '63 and'64)	48.7 ± 1.01	1.50 ± 0.11	60	1.50	+
Red-winged Blackbird Females*('64)	50.8 ± 1.04	1.84 ± 0.13	22	1.81	+
Red-winged Blackbird Males ('64)	76.6 ± 1.04	1.93 ± 0.24	20	1.26	-
Killdeer, Females* (1962,1963)	98.0 ± 2.07	1.69 ± 0.15	29	0.86	+
Killdeer, Males* (1962,1963)	90.2 1.16	1.56 0.16	36	0.86	+

female naturally develops a brood patch and incubates. The sex that develops brood patch in these two species was found to have higher relative pituitary weight. In the Killdeers in which both sexes form brood patches and incubate, relative pituitary weights were similar in both sexes.

Since there are no obvious reasons for sexual difference in adenohypophyseal function in breeding birds of the three species examined other than relative difference in prolactin production, the data can be considered as a supporting indirect evidence for different prolactin contents in the two sexes of Wilson's phalaropes and Redwinged blackbirds, while Killdeer provers probably have similar prolactin content in both sexes.

V. Discussion:

A. Androgens and aggressive as well as sex behavior in phalaropes compared to other birds.

From the literature cited above there is no doubt that in birds androgens are responsible for the aggressive behavior as well as male sex behavior. As mentioned above female phalaropes are known to be more aggressive than the males and play a leading role in the courtship which is usually performed by the males in other common birds during the breeding season. This peculiar feature of female phalaropes was then thought to be in association with the

endogenous ovarian androgens. Our findings that there was relatively higher ovarian content of androgens than that in the testes of conspecific males, and that there was a higher ratio of ovarian testosterone/estradiol than most of the other species examined explain the fact of unusual sexual reversal in female phalaropes.

Conversely, the relatively reduced aggressiveness of male breeding phalaropes could be accounted for by the less production of androgen or it might be more or less influenced by relatively high estrogen production compared to that of the males of other species, as it has been demonstrated that estrogen can somewhat reduce the aggressiveness in birds (7).

The observation that Killdeer ovaries appeared to contain more testosterone than estradiol does not detract from the significance of the relatively high testosterone content of the phalarope ovaries. Firstly, the Killdeer cannot be sexed externally and the roles of the two sexes in aggressive and display behavior are not clear. Secondly, in the closely related Dotterel (a member of the same family as the Killdeer), Thomson (90) has described male Dotterels being chased by the females within the flock and the females make territorial display fights. These observations indicate that in Dotterels as in phalaropes the female is the dominant partner in sexual behavior and this might also apply to the Killdeer.

In contrast to the female phalaropes the breeding ovaries of the Redwinged blackbirds, domestic hen and breeding mallards were found to contain a much larger amount of estradiol than testosterone. In all these birds, the females are known to play a subordinate role in sexual behavior and they show no aggressiveness as do the female phalaropes. In the case of breeding domestic pigeon, the ovarian testosterone was just a little lower than that of the phalaropes. This is not surprising since the female pigeon does not normally differ conspicuously from the male in aggressive behavior.

The findings that testes of breeding cockerel and breeding mallards contained considerably more testosterone than estrogen clearly fit the observed facts that the males of these birds are dominant over the females in both aggressive and sexual behavior. Though the testes of male pigeons did not contain as much androgen as those of the above two species, the testosterone content was still higher than the estrogen content. In the case of Redwinged blackbirds, although testicular testosterone/estradiol ratio was somewhat low it was still much higher than the ovarian testosterone/estradiol ratio, and the total testicular androgen content was still higher than that of estrogens. Hence, the data of the present study on phalarope gonads in comparison with the other species apparently explain the behavioral sexual reversal of these birds as well as

as the normal sexual relationship in the other species.

B. Hormones and Plumage in phalaropes.

As cited in the literature review section, previous studies have shown that gonadal hormones as well as certain gonadotrophins are responsible for the differences between male and female plumage in many different species of birds. Estrogen induces brown Leghorn hen plumage, LH induces cock plumage in the weaver finch and androgens are contributed to the development of cock plumage in the Ruff, Blackheaded gull and Herring gull. Since female phalaropes wear a more colorful plumage than the male at the breeding season and the growth of this plumage is correlated with the onset of aggressive courtship behavior, ovarian androgens were suspected to be the main factor that controls the bright nuptial plumage. The view of androgen control of female nuptial plumage in phalarope has been directly demonstrated by hormonal injection. Johns (64) showed that both male and female Wilson's and Northern phalaropes in the dull fall plumage react to exogenous testosterone but not to other hormones by donning the bright plumage typical of the female nuptial plumage.

Our finding of a relatively high ovarian androgen content in breeding female phalaropes explains the natural occurence of more colorful female nuptial plumage. It is possible that the ovarian testosterone would be even higher at the very onset of breeding season during which the

the nuptial plumage is developed, than the period during which we were able to collect the specimens, since estrogens are believed to be formed from androgens in the course of biosynthesis (91).

One might think that steroid secretion from the adrenals also contributes to the development of the nuptial plumage and the sex difference in aggressive behavior in phalaropes. But it was found that androgen content per unit weight of adrenals was slightly higher in male than in the female Wilson's phalaropes and mallards (92). Also, there is no sex difference in relative adrenal weights in phalaropes. In 85 male phalaropes and 51 females, Höhn et al (93) found that relative adrenal weights on the basis of a standard body weight of 50 g. for both sexes were 7.6 mg for males and 7.5 mg for females. Hence it is unlikely that adrenals play a significant role in the determination of nuptial plumage and sex difference in aggressive behavior.

As was noted above that at the height of breeding season mallard drakes are in the eclipse plumage (or hen like plumage). Castration prevents the development of this hen like eclipse plumage and estrogen but not androgen induces the eclipse plumage in castrates. Our finding of significantly higher estrogen content in the testes of breeding as compared to the post breeding mallards supports the view of estrogen induction of the eclipse plumage in male mallards. It shows the presence

of adequate amounts of testicular estrogen during the breeding season to account for the development of the eclipse plumage.

C. Hormones and incubation patches in phalaropes.

An incubation patch is a naked vascular area on the ventral surface of one or both sexes of birds. It transfers heat from the incubating bird to the eggs during incubation and is considered analogus in its response to hormones to the primitive mammary glands (94). The hormones required for the development of the incubation patch are believed to be quite similar to those required for the development of the mammary gland except that the latter also requires progesterone (95). Bailey has shown that in fringillids, the incubation patch was developed in response to the synergistic action of estrogen and prolactin. The incubation patches produced by these two hormones are similar to those normally occuring in the birds in the breeding season.

In phalaropes, only the males develop incubation patches and incubate. It was suspected that synergestic action of prolactin and androgen instead of estrogen might be responsible for the development of incubation patch in these birds. This hypothesis was tested by Johns and Pfeiffer (10). They found that only testosterone and prolactin in combination produced incubation patches in both male and female Northern and Wilson's

phalaropes.

As we found that the female phalaropes are not deficient in androgen but rather good androgen producers. their failure to form incubation patches was thought to depend on a deficiency of prolactin rather than a lack of androgen production. The results from assays of Wilson's phalarope pituitaries on pigeon crop sac did in fact show that the breeding male phalaropes produce some prolactin while the females produce significantly less or no prolactin. From the histological study, male Northern phalarope pituitaries were shown to contain significantly larger number of acidophils per unit area than those of the females. As was stated above, these acidophils are believed to be mainly prolactinocytes since all the birds examined were fully grown adults which would certainly contain few STH-o-cytes. These observations support the idea of the deficiency of prolactin in the female phalaropes.

In addition, the data on relative pituitary weights of breeding season Wilson's phalaropes, Redwinged blackbirds and Killdeers showed that in the first two species in which only one sex naturally develops an incubation patch and incubates, that sex had the higher relative pituitary weight. In the Killdeers in which both sexes form incubation patches and incubate, the relative pituitary weights were similar in the two sexes. As was mentioned, there is no other obvious reason for sexual differences

in adenohypophyseal function in breeding birds of the three species examined. It is thus believed that the difference in weight of pituitaries of both sexes is due to the difference in prolactin production.

Hence, the data from the study on pituitaries support the view that the failure of incubation patch development in female phalaropes is due to the deficiency or inadequate prolactin production in these birds. The males produce an adequate quantity of prolactin which acts with testicular testosterone for the development of the incubation patch. Prolactin is probably also involved in inducing incubation of the eggs and brooding of the young when they are hatched.

VI. Summary and Conclusions:

The purpose of the present study was to investigate the controlling factors that induce the naturally occurring brighter nuptial plumage in female phalaropes and more active aggressive and courtship behavior in the female than male phalaropes. The cause of the restriction of incubation patch formation and incubation behavior to the male phalarope was also studied.

Five (in some cases six) steroid hormones were separated from the extracts of groups of Wilson's phalarope gonads by means of paper chromatography followed by colorimetric determination. For comparison, similar determina-

tions were carried out on gonadal extracts from five other species of birds. To test the pituitary prolactin content in both sexes of phalaropes, pigeon crop sac tests and histological study were performed. Indirect evidence from relatively different pituitary weights was also noted. It was found that:

(1) The gonadal steroid hormone content reflected the levels of the gonadal secretion.

(2) In comparison with the other birds examined, except the Killdeer, phalarope ovaries showed a high testosterone content, exceeding in four out of six samples the testosterone content per unit body weight of the testes of conspecific males. The ovarian testosterone/estradiol ratio was also higher in phalaropes than in the other species examined.

(3) There was an elevated estrogen content of the testes of breeding as compared to non-breeding mallards.

(4) Results from the pigeon crop sac test showed that male phalaropes produce some prolactin whereas the females produce significantly less or no prolactin.

(5) A higher proportion of acidophils per unit area of adenohypophysis was shown in the male than in female Northern phalaropes collected during the breeding season.

(6) Relatively higher pituitary weights were shown in the sex that naturally develops incubation patch and incubates in Wilson's phalaropes and Redwinged blackbirds.

Similar pituiatry weights were shown in both sexes of Killdeers in which both sexes form brood patches and incubate.

Taken in conjunction with the investigation of other workers that testosterone but not estrogen can induce bright nuptial plumage in phalaropes, the relatively high ovarian testosterone content of phalaropes explains the natural occurrence of brighter plumage and more aggressive behavior in female than the male phalaropes. The high androgen content of Killdeer ovaries suggested that as in phalaropes, the female is the dominant sex in the Kildeer.

The high estrogen content of the testes of breeding mallards supported the hypothesis of estrogen control of the male eclipse plumage in mallards.

Results from the pigeon crop sac tests and histological study explained the development of incubation patches and incubation in male but not in female phalaropes. Supporting indirect evidence came from the observation of sex difference in pituitary weights.

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